

Schwann cell proliferation during Wallerian degeneration is not necessary for regeneration and remyelination of the peripheral nerves: Axon-dependent removal of newly generated Schwann cells by apoptosis

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Peripheral nerve injury is followed by a wave of Schwann cell proliferation in the distal nerve stumps. To resolve the role of Schwann cell proliferation during functional recovery of the injured nerves, we used a mouse model in which injury-induced Schwann cell mitotic response is ablated via targeted disruption of cyclin D1. In the absence of distal Schwann cell proliferation, axonal regeneration and myelination occur normally in the mutant mice and functional recovery of injured nerves is achieved. This is enabled by pre-existing Schwann cells in the distal stump that persist but do not divide. On the other hand, in the wild type littermates, newly generated Schwann cells of injured nerves are culled by apoptosis. As a result, distal Schwann cell numbers in wild type and cyclin D1 null mice converge to equivalence in regenerated nerves. Therefore, distal Schwann cell proliferation is not required for functional recovery of injured nerves.

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Introduction

Injury to the peripheral nervous system (PNS) initiates a sequence of degenerative cellular and molecular changes in the nerve segment distal to the injury site termed "Wallerian degeneration". In rodents, within 24 to 48 h following nerve injury, the distal axons degenerate and the associated Schwann cells break down their myelin sheath. As myelin and axon disintegrate, the denervated

Schwann cells and infiltrating macrophages remove axonal and myelin debris by phagocytosis. This is followed by Schwann cell proliferation, which begins 3 to 4 days after nerve injury. As axons regenerate, they enter the space in distal nerve segments occupied by denervated Schwann cells and subsequently are guided to their original targets. Following re-myelination of the axons and reinnervation, functional recovery of the injured nerve is achieved within 4 to 5 weeks after the initial injury (Fawcett and Keynes 1990; Scherer and Salzer 2003; Waller 1851).

The function of distal Schwann cells that promote nerve regeneration has been well defined. Following injury, denervated Schwann cells begin to produce a number of neurotrophic factors that support the survival of injured neurons (Scherer and Salzer 2003). They also promote macrophage infiltration to the injured nerve (Banner and Patterson 1994; Bolin et al., 1995; Siebert et al., 2000; Toews et al., 1998; Tofaris et al., 2002) and provide a substrate for axonal growth (Araki and Milbrandt 1996; Kleitman et al., 1988; Martini 1994). Continuity of the Schwann cell tube and the extracellular matrix across the injury site allow axons to reinnervate their original targets (Aldskogius et al., 1987; Brown and Hardman 1987; Brushart 1993; Sketelj et al., 1989). Most important, these Schwann cells ensheath and remyelinate regenerating axons.

Schwann cell proliferation during Wallerian degeneration results in a marked increase in Schwann cell number in the distal stump (Thomas, 1948). To address a putative role of Schwann cell proliferation in nerve regeneration, we used a mouse model deficient in cyclin D1, a G1 cell cycle protein. Previously, we and others have shown that distal Schwann cell proliferation during Wallerian degeneration is impaired in mice lacking cyclin D1 (cyclin D1^{-/-}) (Atanasoski et al., 2001; Kim et al., 2000). This defect is specific to proliferation of Schwann cell as the preceding demyelination and dedifferentiation occur normally. In addition, neuronal injury

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responses, including axonal degeneration and regrowth, are not affected in mutant mice (Kim et al., 2000). Therefore, this mouse model is ideal for investigating the consequences of the lack of Schwann cell proliferation during PNS regeneration.

Here, we show that the lack of Schwann cell proliferation in cyclin D1^{-/-} mice does not perturb axonal regeneration and remyelination of the regenerating nerve. Functional recovery of damaged nerves is also achieved normally in cyclin D1^{-/-} mice. In wild type mice, new Schwann cells generated in the distal nerve stumps are removed by apoptosis during a period of Schwann cell proliferation. Our results suggest that Schwann cell proliferation during Wallerian degeneration is not necessary for regeneration and functional recovery of injured peripheral nerves.

Results

Remyelination and ensheathment of regenerated axons occurs normally in the absence of distal Schwann cell proliferation

Distal Schwann cell proliferation following peripheral nerve injury is impaired in mice lacking cyclin D1 (Atanasoski et al., 2001; Kim et al., 2000). Furthermore, short-term axonal regrowth into distal nerve stumps after crush injury occurs normally in the absence of distal Schwann cell proliferation (Kim et al., 2000). To address whether generation of new Schwann cells might be needed for providing long-term stability, proper ensheathment and remyelination of regenerating axons, we compared morphologies of distal axons of wild type and cyclin D1^{-/-} mice 7 weeks following sciatic nerve crush (Fig. 1, top and middle panels). In unlesioned adult sciatic nerves, axonal density and morphology of wild type and cyclin D1^{-/-} mice were indistinguishable from each other, confirming our previous report that peripheral nerves develop normally in cyclin D1^{-/-} mice (top panels) (Kim et al., 2000). Seven weeks following nerve crush, regenerated axons in both wild type and mutant distal nerves were generally smaller in diameter than axons in unlesioned nerves, as expected of regenerating axons. The mean axonal densities (\pm SEM) in the regenerated nerves of wild type and cyclin D1^{-/-} were $46,214 \pm 2,706$ axons/mm² and $45,650 \pm 2,568$ axons/mm², respectively. There was no statistical difference between the two groups ($p > 0.05$) suggesting that lack of distal Schwann cell proliferation did not impair axonal regeneration in cyclin D1^{-/-} mice.

Axonal regeneration is followed by re-myelination of large caliber axons and ensheathment of small caliber axons by distal Schwann cells. To determine whether Schwann cells and regenerated axons were associated properly in the absence of newly generated Schwann cells, we compared morphologies of myelinating- and non-myelinating Schwann cells in wild type and cyclin D1^{-/-} distal nerves 7 weeks after sciatic crush. As shown in electron micrographs (Fig. 1, bottom panels), myelinated axons of mutant distal nerves appeared morphologically normal and stable, indistinguishable from wild type: myelin sheaths are compacted without separation and the myelin thickness appeared normal. The non-myelinating Schwann cells also appeared normal as small caliber axons were properly segregated into bundles surrounded by Schwann cell cytoplasm. This result indicates that ensheathment and remyelination of regenerated axons occur normally in cyclin D1^{-/-} nerve despite the lack of earlier Schwann cell proliferation. The result also suggests that the number of non-dividing pre-existing Schwann cells is sufficient to establish proper axon–Schwann cell units in the regenerated nerve.

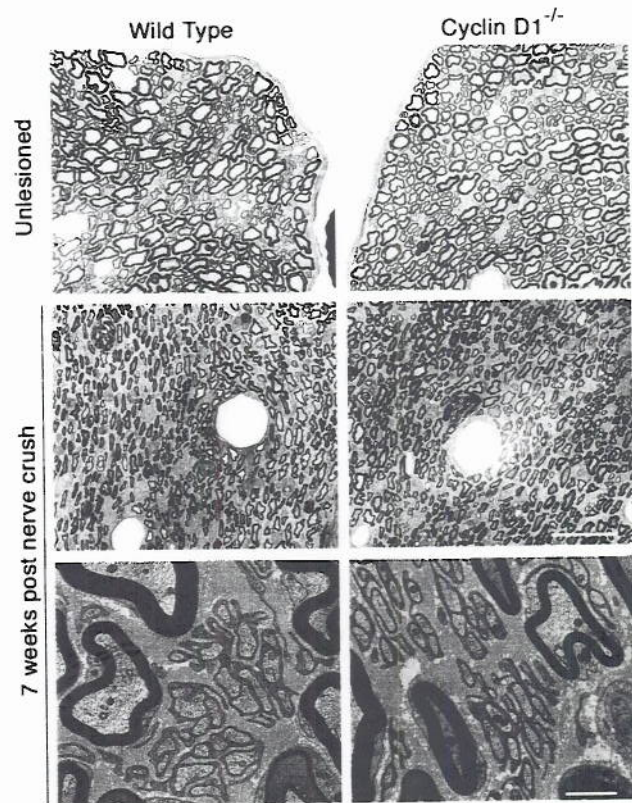


Fig. 1. Morphology of the distal sciatic nerves of wild type and cyclin D1^{-/-} mice. Top panels: Semi-thin transverse sections of unlesioned sciatic nerves of wild type and cyclin D1^{-/-} mice. Middle panels: Seven weeks following sciatic nerve crush. Regenerated axons are smaller in diameter than axons in the unlesioned nerve. Axonal density and morphology of cyclin D1^{-/-} nerve do not appear to be different from wild type nerve. Scale bar: 60 μ m. Bottom panels: Ultra-thin transverse sections are shown in order to visualize myelinated and non-myelinated axons of the regenerated sciatic nerve. Large axons are myelinated normally and small axons within Remak bundles are well segregated by Schwann cell plasma membrane in both groups. Scale bar: 20 μ m.

Sciatic nerve function recovers normally in the absence of distal Schwann cell proliferation

Next, we addressed whether the Schwann cell proliferation might be required for long-term functional recovery of injured sciatic nerve. Walking track analysis was performed on wild type and cyclin D1^{-/-} mice at 1, 7, 14 and 21 days post sciatic nerve crush, and nerve function was evaluated by determining the sciatic functional index (SFI) as described previously (Inserra et al., 1998). Preoperative analysis was also performed to determine normal SFI value for each animal (Fig. 2). Prior to the injury, cyclin D1^{-/-} mice exhibited an average SFI value of -23.1 , which was lower than the SFI of -3.8 obtained from the wild type mice. This is probably due to the fact that cyclin D1^{-/-} mice are smaller in size and often exhibit difficulties in walking and balancing themselves (Sicinski et al., 1995). Immediately following sciatic nerve crush, SFI values dropped to -95.3 and -85.0 in wild type and cyclin D1^{-/-} mice, respectively, indicating loss of sciatic nerve function. The nerve function then recovered gradually in both groups, indicated by an increase in SFI values. By 21 days, the SFIs of both groups reached preoperative levels. No significant delay in nerve function recovery

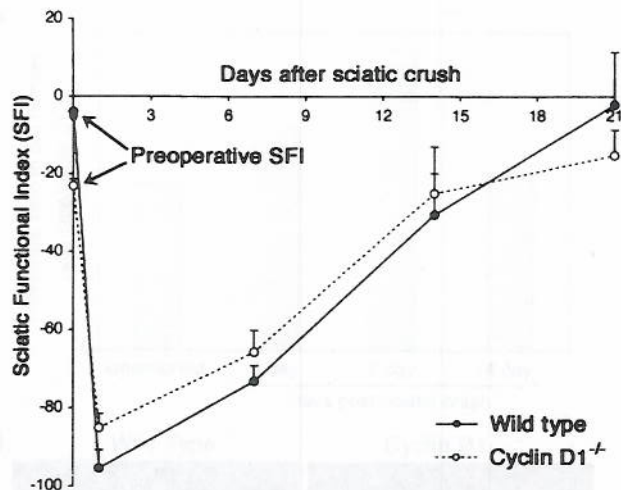


Fig. 2. Sciatic nerve function recovers normally in the absence of distal Schwann cell proliferation in cyclin D1^{-/-} mice. Sciatic nerve function was evaluated by determining Sciatic Function Index (SFI) from parameters obtained from walking track analysis before (preoperative SFI) and after sciatic nerve crush. Soon after nerve crush SFI in both animals decreased to levels near -100, representing complete loss of function. The SFI recovered to the preoperative values by 3 weeks in both groups. No significant difference in the recovery rate was observed between wild type and cyclin D1^{-/-} mice. The means (\pm SEM) were calculated from four animals per group.

was observed in cyclin D1^{-/-} mice when compared to the wild type. We also tested sensory function recovery by performing a paw-withdrawal test upon electrical stimulation on the mouse footpads: no significant impairment or delay was observed in cyclin D1^{-/-} mice (not shown). These results suggest that the lack of Schwann cell proliferation in cyclin D1^{-/-} mice does not impair or result in delayed recovery of sciatic nerve function.

Distal Schwann cell proliferation in wild type mice does not result in the appearance of excess Schwann cells associated with regenerated axons

Lack of Schwann cell proliferation in cyclin D1^{-/-} distal nerve stumps predicts that in wild type littermates, the distal Schwann cell proliferation should result in generation of excess Schwann cells associated with regenerated axons. To investigate this, we compared average lengths of myelin segments associated with regenerated axons in wild type and cyclin D1^{-/-} nerves. We reasoned that excess Schwann cells or an increase in Schwann cell number associated with individual axons in wild type should result in the formation of shorter myelin segments. Teased nerve fibers were prepared from distal sciatic nerves of wild type and cyclin D1^{-/-} mice at 6 weeks after nerve crush, and myelin segment length was determined by the distance between two adjacent nodes (internodal length) along an axon (Fig. 3). In both wild type and cyclin D1^{-/-} mice, myelin segments on regenerated axons were shorter than myelin segments formed on unlesioned axons prior to nerve injury, confirming previous reports on myelination of regenerated axons (Hildebrand et al., 1985). When myelin segment lengths on regenerated axons of wild type and cyclin D1^{-/-} mice were compared, the average length of segments was $300.9 \pm 13 \mu\text{m}$ and $276.3 \pm 20.3 \mu\text{m}$ in wild type and cyclin D1^{-/-} mice, respectively, not significantly different from each other. This result suggests that Schwann cell numbers associated

with regenerated axons are similar in wild type and mutant mice, hence injury-induced Schwann cell proliferation in wild type does not result in presence of excess Schwann cells associated with regenerated axons.

The difference in distal Schwann cell number between wild type and cyclin D1^{-/-} mice diminishes as peripheral nerve regeneration progresses

Despite the earlier increase in Schwann cell number following injury in wild type mice, we observed that Schwann cell number on regenerated axons was not significantly different between wild type and cyclin D1^{-/-} mice. Consequently, this suggests that in adult animals, the number of axon-associated Schwann cells is regulated during PNS regeneration. To address this, we compared wild type and cyclin D1^{-/-} distal cell numbers at different times after sciatic crush. We reasoned that if such regulatory mechanisms exist in adult animals, Schwann cell numbers in wild type and mutant mice should equilibrate over time. In other words, the initial difference in distal Schwann cell number should diminish as the regeneration progressed. The data are represented as the numerical ratio between distal cell number obtained from wild type and cyclin D1^{-/-} mice (Fig. 4A). In control unlesioned sciatic nerves, the differential ratio was close to 1, indicating that there was no significant difference in Schwann cell number between wild type and cyclin D1^{-/-} nerve. On day 5 after sciatic crush, wild type distal cell number was an average of 1.4-fold higher than that

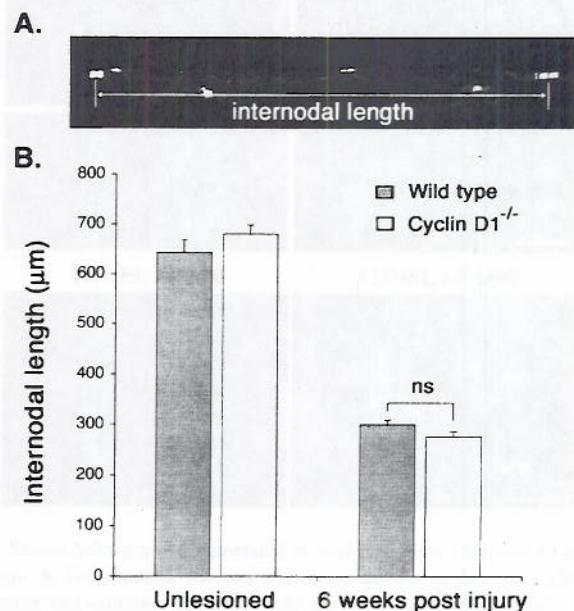


Fig. 3. Myelin segments on regenerated axons are similar in length in wild type and cyclin D1^{-/-} mice. A. Length of a myelin segment was determined by measuring the internodal length. Teased nerve fibers were prepared and immunostained for a paranodal protein Caspr. Internodal length is determined by the distance between two nodes along an axon, with each node encompassed by Caspr⁺ paranodes. B. Comparison of average myelin segment lengths associated with unlesioned or regenerated axons (6 weeks following sciatic nerve crush) between wild type and cyclin D1^{-/-} mice. The difference between the two groups is not statistically significant (ns). The means (\pm SEM) were calculated from analyzing 80–120 myelin segments from three animals per group. Statistical significance was determined by ANOVA test.

cell numbers in wild type and cyclin D1^{-/-} mice eventually converge to equivalence on regenerated axons.

Excess Schwann cells are removed from wild type distal nerve by apoptosis

One of the mechanisms by which the distal cell numbers between wild type and cyclin D1^{-/-} mice could equalize is removing excess Schwann cells from the wild type nerve. A similar observation has been made in developing peripheral nerves following a period of Schwann cell proliferation in which the final Schwann cell number is determined in an axon-dependent manner: excess Schwann cells without axonal contact are removed by apoptosis (Grinspan et al., 1996; Nakao et al., 1997; Syroid et al., 1996). To investigate this, TUNEL assay was performed on distal nerve sections from wild type and cyclin D1^{-/-} mice to look for the presence of apoptotic cells (Fig. 5). Five days after nerve crush, a significant number of apoptotic nuclei were observed in wild type distal nerve, whereas only a few apoptotic cells were detected in cyclin D1^{-/-} nerve (Fig. 5A). Double immunofluorescence to label TUNEL-positive nuclei with Schwann cell-specific protein S100 or macrophage marker F4/80 antigen clearly showed that most of the apoptotic cells were Schwann cells, although a few apoptotic macrophages were also observed (Fig. 5C). To further characterize the progression of Schwann cell death during PNS regeneration, distal nerve sections collected at 3, 5 and 14 days after sciatic crush were subjected to TUNEL assay and the percentages of apoptotic nuclei were determined (Fig. 6). Between 3- and 5-day post-crush, the percentage of apoptotic cells in wild type nerves ranged from 7% to 8%, whereas the percentage in cyclin D1^{-/-} nerves remained below 3%. A second spike of apoptosis was seen 14 days post-crush in wild type, with approximately a two-fold increase in the number of apoptotic cells when compared to the earlier apoptotic period. This might reflect the death of Schwann cells that accompanies the second phase of Schwann cell proliferation that occurs when more regenerating axons grow into the denervated nerve stump (Pellegrino and Spencer 1985). A slight increase in apoptosis was also seen in the mutant nerves, however to a much lower level than

wild type. This is likely to represent the death of macrophages as it has been shown that peak apoptosis of invading macrophages occurs between 10 and 15 days after nerve injury in mice (Kuhlmann et al., 2001). The same result was obtained when apoptosis was detected by immunostaining using an antibody directed against small poly (ADP-ribose) Polymerase 1 (PARP) fragment, a cleavage product generated by caspases during apoptosis (data not shown). This result suggests that excess Schwann cells generated in wild type are removed by apoptosis, whereas most of the non-dividing denervated Schwann cells in the cyclin D1^{-/-} survive.

During development, axons provide survival signals for the associated Schwann cells (Dong et al., 1995). To determine whether the survival of adult denervated Schwann cells is also dependent on axonal contact, we performed nerve axotomy on wild type and cyclin D1^{-/-} sciatic nerve and determined the proportion of apoptotic cells in the distal nerve. Unlike the nerve crush injury, nerve axotomy prevents proximal axons from re-growing into the distal nerve and therefore, there are no axons to support Schwann cell survival in the region. If the Schwann cell survival requires axonal contact, the percentage of apoptotic Schwann cells in cyclin D^{-/-} mice should increase to the wild type level upon axotomy, in the absence of axonal regeneration. As expected, there was a drastic increase in the number of apoptotic Schwann cells in cyclin D1^{-/-} nerve at both 3 and 5 days following axotomy to a level similar to the wild type (Figs. 5A and 6). To confirm the presence and absence of regenerated axons in distal nerve stumps after nerve crush and nerve axotomy, respectively, distal nerve sections of wild type and cyclic D1^{-/-} mice were collected at day 5 following injury and immunostained for GAP-43, a protein expressed in growing axons (Fig. 5B). GAP-43⁺ regenerating axons were present in both wild type and mutant distal nerve stumps following nerve crush but not axotomy. This result suggests that the survival of adult denervated Schwann cells during PNS regeneration is dependent on the presence of regenerating axons. Interestingly, however, the rate of Schwann cell apoptosis diminished significantly on day 7 in both wild type and cyclin D1^{-/-} mice, suggesting that the distal Schwann cell death did not continue indefinitely. This result indicates that the distal environment is capable of supporting minimal Schwann cell survival in the absence of regenerating axons. In

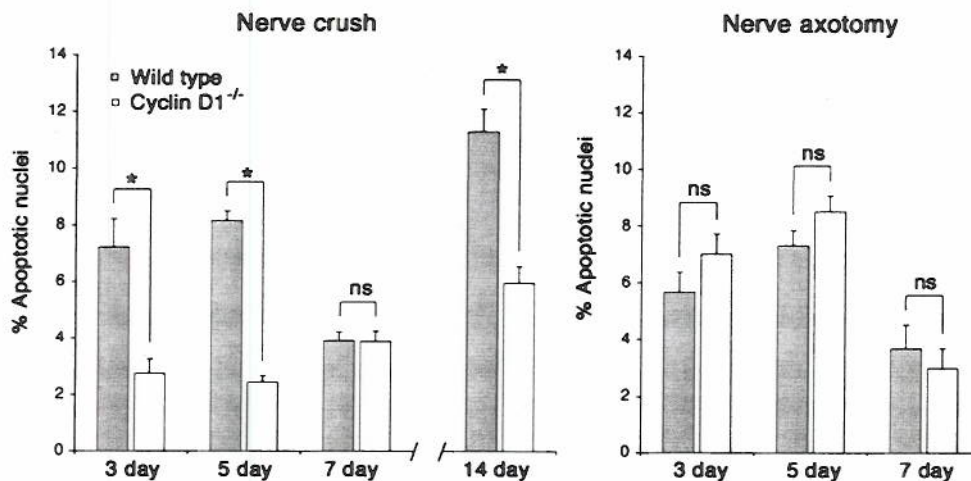


Fig. 6. Survival of distal denervated Schwann cells is dependent on the presence of regenerating axons. Distal nerve sections from wild type and cyclin D1^{-/-} sciatic nerves prepared at 3, 5, 7 and 14 days following nerve crush, or 3, 5, 7 days following nerve axotomy were processed for detection of apoptosis by TUNEL assay. The data represent the mean percentage of apoptotic cells (\pm SEM) in 30–50 fields obtained from three animals. Statistical significance was determined by ANOVA test. *: $p < 0.0001$, ns: not significant.

summary, as peripheral nerve axons regenerate, surplus Schwann cells generated during the Schwann cell proliferation period are removed in an axon-dependent manner. This is probably one of the mechanisms by which Schwann cell and axon numbers are correctly established in distal nerves during adult peripheral nerve regeneration.

Discussion

In this study we have sought to answer the question of whether proliferation of denervated Schwann cells plays a significant role during PNS regeneration. By comparing nerve regenerative properties in wild type and cyclin D1^{-/-} mice, we report that Schwann cell proliferation in the distal nerve is not necessary for re-myelination and functional recovery in adult mice. We also show that excess Schwann cells generated in wild type nerve are removed by apoptosis during the proliferative period of the Schwann cells. When axonal regeneration is prevented by axotomy, Schwann cells in both wild type and cyclin D1^{-/-} mice undergo apoptosis, suggesting that the survival of adult denervated Schwann cells is dependent on axonal regeneration.

The Schwann cell-specific phenotype of cyclin D1^{-/-} mice enables us to examine the direct consequences of the lack of Schwann cell proliferation during PNS regeneration. In these mice, Schwann cell response to nerve injury occurs normally except that they do not proliferate. More important, other cellular responses associated with PNS injury including neuronal immediate early gene responses, axonal degeneration and growth are not affected (Kim et al., 2000). In the present study, by comparing the rate of nerve function recovery between the wild type and cyclin D1^{-/-} mice, we have shown that a lack of Schwann cell proliferation does not hinder functional recovery of the sciatic nerve following nerve crush, indicating that the generation of new Schwann cells in the distal nerve is not necessary for restoring PNS nerve function. Walking track analysis is a widely used method for evaluating sciatic nerve function. However, we cannot rule out a possibility that the test might not have been sensitive enough to monitor a slight delay in normal physiological recovery of nerve activity. Direct measurement of nerve conduction velocity on the regenerating nerve would provide more detailed information at the electrophysiological level. However, the invasive nature of the method would not be appropriate for use on cyclin D1^{-/-} mice, which are in general weaker and smaller than their wild type littermates (Sicinski et al., 1995).

Ultra-structural analysis on the regenerated distal nerve of cyclin D1^{-/-} mice did not show any morphological abnormalities in myelinated and non-myelinated axons when compared to the wild type. Earlier studies have elucidated the importance of distal Schwann cell proliferation during PNS regeneration and remyelination. (Hall and Gregson, 1974) have shown that when Schwann cell proliferation that follows lyssolecithin-induced demyelination is blocked by intraneural injection of an anti-mitotic drug mitomycin C, the subsequent remyelination of the lesion site is delayed: noted by axons that are incompletely surrounded by Schwann cell cytoplasm or in the absence of associated Schwann cells. We did not find such defects in regenerating cyclin D1^{-/-} nerve. In a similar study, (Pellegrino and Spencer, 1985) have shown that pre-treatment of denervated distal nerve with mitomycin C prior to reinnervation delays regeneration and remyelination, suggesting that Schwann cell proliferation plays an important role in promoting remyelination and regeneration of the injured PNS. The discrepancy between our result and these earlier studies might be explained by a possible cytotoxic effect of mitomycin C on regenerating axons or on the associated

Schwann cells that would affect remyelination and regeneration. Although these studies were based on the notion that mitomycin C acted primarily by inhibiting Schwann cell division, a later study has shown that the drug also induces Schwann cell demyelination and death when injected into normal sciatic nerve (Westland et al., 1990).

Despite the increase in distal Schwann cell number after injury in wild type mice, we did not observe any signs of excess Schwann cells associated with distal axons once regeneration was complete. Analysis of internodal length of remyelinated segments and counting of distal Schwann cell numbers indicated that Schwann cell numbers in the wild type and cyclin D1^{-/-} mice were equalized to a similar level. In a recent report, Murinson et al. (2005) have studied the fate of distal proliferating Schwann cells following transection of myelinated motor fibers in rat sciatic nerve using electron microscopy combined with *in vivo* [³H]-thymidine labeling. It was shown that the number of Schwann cells associated with regenerating axons does not increase significantly despite an increase in the mitotic index, suggesting that excess Schwann cells disappear from the Schwann cell tubes. From the observation it was suggested that as a Schwann cell divides, one Schwann cell remains associated with an axon and the other either leaves the Schwann cell tube or dies due to apoptosis in the absence axonal contact. A similar observation was made in an earlier study of Schwann cell proliferation during focal demyelination (Griffin et al., 1990). In this study, they reported the appearance of many supernumerary Schwann cells with scanty cytoplasm and wrinkled nuclei, containing extensive heterochromatin suggesting regressive change or cell death. These observations are in agreement with our result of an equivalent Schwann cell number in wild type and cyclin D1^{-/-} mice. One could speculate that newly generated Schwann cells in the wild type, once they leave the Schwann cell tubes, die of apoptosis due to the lack of axonal contact. On the other hand, most non-dividing Schwann cells in cyclin D1^{-/-} mice remain within the tubes and survive.

Our data clearly shows Schwann cell death by apoptosis following nerve injury in adult mice, which has also been reported by other groups (Ekstrom, 1995; Ferri and Bisby, 1999; Lino et al., 2007; Triolo et al., 2006). This is in contrast with an earlier study by (Grinspan et al., 1996) showing that axotomy-induced Schwann cell apoptosis was rarely detected in adult rat sciatic nerve. It is unlikely that the newly generated Schwann cells in rat distal nerves continue to remain within the Schwann cell tubes in the absence of regenerating axons because it has been shown that Schwann cells disappear from the distal segments of chronically denervated rat nerves (Weinberg and Spencer, 1978). Since it is possible that the difference in the sensitivity of the apoptosis detection methods could have resulted in the discrepancy between rat and mouse models, we conducted a TUNEL assay simultaneously on rat and mouse adult sciatic nerves prepared 5 days following axotomy. No apoptotic Schwann cells were found in the rat, whereas numerous apoptotic Schwann cells were detected in the mouse nerve (data not shown). One possible explanation for the lack of detectable apoptotic Schwann cells in the adult rat PNS is that Schwann cells migrate out of the Schwann cell tubes and subsequently lose their Schwann cell phenotypic marker (Murinson et al., 2005). A fate determination study using *in vivo* live-cell labeling and imaging techniques should address this issue. Another possibility, which is more plausible, is that excess Schwann cells are removed by necrosis or by apoptosis in the absence of DNA fragmentation, in which case the death would not be detected by TUNEL assay. Supporting the latter, it has been shown that rat Schwann cells isolated from the degenerating sciatic nerve undergo cell death

with typical cytoplasmic changes associated with apoptosis, but without DNA degradation (Hirata et al., 1998, 2001). A similar apoptotic mechanism has been demonstrated in other cell types (Falcieri et al., 1993; Ucker et al., 1992).

Immunofluorescence on distal nerve sections showed that Schwann cells undergoing apoptosis were S100⁺, indicating that they were similar to that of immature Schwann cells or premyelinating Schwann cells. Unlike Schwann cell precursors (S100⁻), S100⁺ Schwann cells survive in culture in the absence of axonal contact owing to the autocrine loop (Cheng et al., 1998; Dowsing et al., 1999; Meier et al., 1999). However, our data indicates that the S100⁺ cells found in adult PNS following nerve injury are susceptible to death in the absence of axonal contact. A possible mechanism of S100⁺ Schwann cell death in adult mice is the up-regulation of the p75NGFR expression following axotomy (Heumann et al., 1987). A role of p75NGFR in mediating cell death has been implicated in several cell types (Barrett and Bartlett, 1994; Casaccia-Bonnett et al., 1996; Frade et al., 1996; Rabizadeh et al., 1993; Syroid et al., 2000). Supporting this, the lack of p75NGFR has been shown to significantly block axotomy-induced Schwann cell death both in neonatal and adult mice (Ferri and Bisby, 1999; Syroid et al., 2000).

Myelin segment lengths on remyelinated axons are in general shorter than the segments on normal nerves formed during development (Hildebrand et al., 1985). The myelin shortening has been regarded as a result of Schwann cell crowding following the cell proliferation after nerve injury or demyelination. However, in our study, we observed a similar shortening of the myelin segments in regenerated fibers of cyclin D1^{-/-} mice despite the absence of distal Schwann cell division, suggesting that it is independent of Schwann cell proliferation. Interestingly, when we compared the actual Schwann cell densities (\pm SEM) on the unlesioned and regenerated nerve fibers of cyclin D1^{-/-} mice, to our surprise, there was a significant increase in the Schwann cell number associated with the regenerated axons; 1248.0 \pm 43.5 cells/mm² and 1883.9 \pm 53.9 cells/mm² for unlesioned and regenerated nerves, respectively ($p < 0.05$). The mechanism by which extra Schwann cells appear in distal stumps in the absence of Schwann cell proliferation is unknown. One possible mechanism is migration of Schwann cells from the proximal nerve stumps; it has been shown previously that when axons are allowed to grow into distal nerve stumps devoid of Schwann cells, the regenerating axons are accompanied by Schwann cells migrating from the proximal region of the injury (Sketelj et al., 1989).

Why do Schwann cells proliferate in the distal nerve stump following nerve injury? During development, generation of excess cells is a common strategy to ensure that there will be sufficient cells for organization of a particular tissue. Once produced, the excess cells are removed. The Schwann cell response to nerve injury in adult animals recapitulates the developmental process. Distal Schwann cell proliferation followed by induction of apoptosis ensures that Schwann cell and axonal numbers are correctly matched as the damaged nerves are repaired. As in the developing PNS, regulation of the adult Schwann cell number in regenerating nerves is also a tightly controlled event that is accomplished by the positive selection of surviving Schwann cells by the regenerating axons.

Experimental methods

Antibodies

For immunofluorescence, polyclonal antibody to S100, a Schwann cell marker (DAKO, Carpinteria, CA) and macrophage-specific F4/80 antigen

(Serotec, Raleigh, NC) was used at a dilution of 1:150 and 1:10, respectively. A sheep antibody against rat GAP-43 (gift of Dr. Larry Benowitz at Harvard Medical School, MA) was used at a dilution of 1:3000. Monoclonal antibody to Caspr was a gift from Dr. Matthew Rasband (Baylor College of Medicine, TX) and was used at a dilution of 1:200.

Mouse sciatic nerve injury

Mice were anesthetized, and sciatic nerves were exposed at approximately 0.5 cm distal to the sciatic notch. The nerve was crushed once for 30 s using hemostatic forceps, and again for 30 s at the same site but orthogonal to the initial crush. The crush site was marked by loosely placing a silk suture around the crush lesion and the wound was closed. Alternatively, axotomy was performed by transecting the nerve. Unlesioned negative controls were produced by exposing the nerve but leaving it uninjured.

Walking track analysis: Sciatic Functional Index (SFI)

All animals underwent preoperative and postoperative walking track analysis in a fashion described previously for measuring SFI in mouse (Inserra et al., 1998). Briefly, the hind paws of each animal were moistened with water-soluble, non-toxic paint and the animals were allowed to walk unassisted along a 6 \times 44 cm corridor lined with white paper. Prints for measurements were chosen at a point when the mouse was walking at a moderate pace. The tracks were evaluated for two different parameters: toe spread (TS), the distance between first and fifth toes and print length (PL), the distance between the third toe and the hind pad. Measurement for each parameter was made for the right (N: normal) and the left (E: experimental) paw prints. Using the parameters, preoperative and postoperative SFI for each animal was determined by the formula $118.9 \times \{(ETS - NTS)/NTS\} - 51.2 \times \{(EPL - NPL)/NPL\} - 7.5$ as described previously (Inserra et al., 1998).

Electron microscopy and morphometry

Seven weeks following nerve crush injury, the mice were sacrificed and the distal sciatic nerves were harvested and fixed in 4% paraformaldehyde and 2.5% glutaraldehyde in PBS (pH7.4) overnight, washed in PBS, and post-fixed in 2% osmium tetroxide for 1 h. They were then dehydrated in ethanol and embedded in epoxy resin. Eighty nanometer ultra-thin sections were cut and counterstained with uranyl acetate and Reynold's lead citrate. Micrographs were taken with a JEOL 12000EX electron microscope. For morphological analysis, 1 μ m semi-thin sections of epoxy-embedded sciatic nerves were stained with toluidine blue and viewed under light microscopy. Axonal density (the number of myelinated axons per 1 mm²) was determined at a 400 \times total magnification from 8 representative fields collected from three animals per genotype.

Measurement of myelin segment length

Teased nerve fibers were prepared from normal and distal nerve stumps 3 weeks following crush injury from wild type and cyclin D1^{-/-} mouse sciatic nerves as described (Guertin et al., 2005). Briefly, nerves were fixed in 4% paraformaldehyde for 10 min, washed in Tris-buffered saline (TBS) and the perineurium was removed. Nerves were teased first with a 30-gauge needle and then with a sharpened Tungsten needle. A few teased fibers were transferred into a drop of TBS on a coated glass slide and pulled to stretch them out on the slide. Nerves were dried overnight at room temperature and stored at -80 $^{\circ}$ C until ready to use for immunostaining. To visualize paranodes, fibers were immunostained for Caspr. Briefly, teased nerve fibers were re-hydrated in TBS for 3 min then fixed in 4% paraformaldehyde for 20 min. After washing with TBS, samples were permeabilized in blocking solution (5% normal goat serum + 0.1% Triton X-100) for 30 min

and then incubated with Caspr antibody. After washing with TBS, samples were incubated with secondary antibodies for 45 min. Nuclei of cells were visualized by staining with DAPI. Caspr⁺ fibers were photographed and digitized images were analyzed using Metamorph (MDS Analytical Technologies). Individual nodes were identified by a gap flanked by two Caspr⁺-paranodes. Subsequently, a myelin segment length was determined by the internodal length measured as a distance between two adjacent nodes along an axon. A total of 90–120 segments from three animals were counted, and the mean internodal length was determined using StatView software. Significance was determined using the ANOVA test, with a *p*-value less than 0.05 being significant.

DeadEnd™ fluorometric TUNEL and immunofluorescence staining

Distal nerves were harvested and fixed by immersion in 4% paraformaldehyde for 2 h. Longitudinal frozen sections of the nerves ranging from 10 to 12 μm were prepared and processed for immunohistochemistry. For TUNEL staining, the manufacturer's protocol (Promega) was followed. Briefly, sections were fixed in 4% paraformaldehyde in PBS for 15 min, permeabilized in 0.2% Triton X-100 solution for 15 min, and fixed again in 4% paraformaldehyde in PBS for 5 min. Sections were then treated with nucleotide mix in TdT incubation buffer (22.5 μL Equilibration Buffer, 2.5 μL Nucleotide Mix, and 0.5 μL TdT Enzyme per section) for 1 h at 37 °C in a humidified chamber. For S100/TUNEL or F4/80/TUNEL double immunofluorescence, sections were fixed in 4% paraformaldehyde in PBS for 20 min at room temperature, incubated for 1 h with rabbit anti-S100 or F4/80 antibody in PBS containing 5% normal goat serum and 0.2% Triton X-100 solution. After three extensive washes in PBS, sections were incubated with Cy3-conjugated goat anti-rabbit secondary antibody for 1 h at room temperature. Sections were again fixed in 4% paraformaldehyde in PBS for 20 min at room temperature, and then processed for TUNEL staining starting from incubation with the nucleotide mixture. Nuclei were visualized by one-minute incubation with DAPI in PBS (1:1000).

For quantification, photographs were taken of the DAPI-stained nuclei as well as the TUNEL-labeled nuclei at 40× magnification, then overlaid together. The total number of nuclei (ranging from 130–200) was counted per field via DAPI stain and the total number of apoptotic nuclei was counted via overlay staining of TUNEL and DAPI; the resulting percent of apoptotic cells was calculated per field of view. From three animals, a total of 50 fields were counted for each condition. The mean percent of apoptotic cells for each condition was determined using StatView software. Significance was determined using the ANOVA test, with a *p*-value less than 0.05 being significant.

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